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Characterization of an Unidentified *Sarcocystis falcatula*-like Parasite from the South American Opossum, *Didelphis albiventris* from Brazil

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ABSTRACT. An unidentified isolate of a *Sarcocystis falcatula*-like parasite was obtained from the lungs of budgerigars (*Melopsittacus undulatus*) fed sporocysts from a naturally-infected South American opossum, *Didelphis albiventris* from Brazil. Four captive budgerigars fed sporocysts from the opossum intestine died of acute sarcocystosis 8, 10, and 12 days after oral inoculation (DAI); one budgerigar was killed 12 DAI when it was lethargic. Schizonts and merozoites found in the lungs of the budgerigars reacted mildly with polyclonal *S. falcatula* antibody. The parasite was isolated in equine kidney cell cultures inoculated with lung tissue from a budgerigar that was killed 12 DAI. Two budgerigars inoculated subcutaneously with 100,000 culture-derived *S. falcatula* merozoites developed acute sarcocystosis and *S. falcatula*-like schizonts were found in their lungs 15 and 16 DAI. Four budgerigars kept as unfed controls in the same environment remained free of *Sarcocystis* infection. The parasite underwent schizogony in African green monkey kidney cells and bovine turbinate cells. Merozoites divided by endopolygeny, often leaving a residual body. Polymerase chain reaction studies using primers JNB33/JNB54 and Hinf I and Dra I digestion indicated that the isolate was not *S. falcatula.* Results of this study indicated that the South American opossum, *D. albiventris,* is a definitive host for yet another *S. falcatula*-like parasite.

Key Words. Budgerigars, cell culture, development, polymerase chain reaction, schizonts.

SARCOCYSTIS species have a two-host life cycle with herbivores as intermediate hosts and carnivores as definitive hosts. Most species of *Sarcocystis* are host specific for their intermediate hosts but not for the definitive host (Dubey, Speer, and Fayer 1989). *Sarcocystis falcatula* uses avian species as intermediate hosts and the North American opossum (*Didelphis virginiana*) as the definitive host (Box and Duszynski 1978; Box, Meier, and Smith 1984). It is an unusual species of *Sarcocystis* because of its wide intermediate-host range (Passeriformes, Psittaciformes, and Columbiformes), its prolonged schizogony (lasting up to 5 mo), and because of its high pathogenicity to its intermediate host (Box and Smith 1982; Clubb and Frenkel 1992; Smith et al. 1987a, 1987b, 1990a, 1990b). Outbreaks of sarcocystosis in aviaries and zoos are relatively common in the United States (Clubb et al. 1986; Hillyer et al. 1991; Jacobson et al. 1984; Smith et al. 1990b). We have not found reports of natural cases of *S. falcatula* infections from birds residing outside the United States.

Recently, *S. falcatula* was reported for the first time from Argentina (Dubey et al. 1999b, 2000). The present study reports *S. falcatula*-like infection from *Didelphis albiventris* from Brazil for the first time and provides evidence that the parasite is different from *S. falcatula.*

MATERIALS AND METHODS

***Sarcocystis* sporocysts and infection of animals.** *Sarcocystis* sporocysts were obtained from one of three adult opossums (*Didelphis albiventris*) trapped on the University Campus, Jaboticabal, Brazil. The opossums were killed humanely and the epithelium from the small intestine was scraped, homogenized in a blender, and digested in 5.25% sodium hypochlorite (bleach) for 30 min. After repeated centrifugations to remove chlorine, the sporocysts were suspended in antibiotic saline containing 10 mg streptomycin, 10,000 Units penicillin, 500 units myostatin, and 0.05 mg fungizone per ml of Hanks' balanced salt solution (HBSS) (Leek and Fayer 1979) and transported by air to the Beltsville Agricultural Research Center (BARC), where sporocysts were stored at 4 °C.

Sarcocystis sporocysts from opossum were bioassayed in birds and mice. For testing infectivity of sporocysts, 10 to 20-wk-old budgerigars raised in captivity were obtained from a local aviary (Dubey and Lindsay 1998). The sporocysts were deposited in the crops of 4 birds (No. 65, 69, 83, 84) via a feeding needle. Two uninoculated birds (No. 89, 90) were kept as controls. The birds were housed in an isolation building and were given sterilized water and bird feed. The same inoculum was fed to 2 gamma interferon-knockout (KO) mice as described (Dubey and Lindsay 1998, 1999; Dubey, Speer, and Lindsay 1998).

Birds or mice that died or were killed were examined at necropsy. Samples of brain, tongue, heart, lung, liver, spleen, intestines, and pectoral and leg muscles were fixed in 10% buffered neutral formalin. Paraffin-embedded sections were cut at 5 μm and examined after staining with hematoxylin and eosin (HE). Impression smears of lung tissue were examined after staining with Giemsa. Smears of lungs were also made in aqueous saline (0.85% NaCl) and examined microscopically without staining.

For immunohistochemical staining, paraffin sections were reacted with anti-*S. falcatula* antibodies raised in rabbits as described by Dubey and Lindsay (1998). Tissues from experimentally infected birds and mice (Dubey and Lindsay 1999) were used as positive and negative controls. A 1:1000 dilution of anti-*S. falcatula* serum and the peroxidase-anti-peroxidase method (Marsh et al. 1997) were used. Polyclonal anti-*S. neurona* (Dubey et al. 1999a) and anti-*S. speeri* (Dubey and Lindsay 1999) sera were used for immunohistological examination of KO mice tissues.

In vitro cultivation of merozoites and infectivity to birds. Lung from bird No. 84 that was killed 12 d after inoculation (DAI) with sporocysts, was homogenized in RPMI 1640 cell culture medium and inoculated on equine kidney (EK) cells (Dubey et al. 1999) and incubated for 30 min. The homogenate was removed and and replaced with new medium. Merozoites from the culture medium (1×10^5 per bird) were inoculated subcutaneously (s.c.) into 2 birds (No. 98, 100). Two birds (No. 97, 99) served as uninoculated controls. Bird No. 100 was killed 14 DAI and its lungs were used for in vitro cultivation of parasites.

The lungs of birds No. 100 were teased apart with a 27-gauge needle and passed through an 18-gauge needle. The homoge-

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nate was inoculated on African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, MD) and incubated for 30 min. After 30 min the homogenate was removed and cell culture medium was added. The CV-1 cells were grown in plastic cell culture flasks in growth medium consisting of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 100 U penicillin G/ml, and 100 mg streptomycin/ml. Cell cultures were maintained in growth medium in which the FBS content was lowered to 2%. Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Merozoites from cell culture derived from the lung of bird No. 100 were inoculated intramuscularly (i.m.) into 6 birds (No. 149–154). For this, merozoites were counted and 10-fold dilutions were made in tissue culture medium. Two birds were inoculated with 3 dilutions containing 10^7, 10^5, and 10^3 merozoites.

In vitro development of protozoa. For descriptive studies, merozoites were harvested from infected cell cultures by removing the medium and replacing it with HBSS without calcium and magnesium. The CV-1 cells with parasites were removed from the flask, passed through a 27-gauge needle, and filtered through a sterile 3-μm filter to remove cellular debris. Merozoites were counted on a hemacytometer and inoculated on to 22-mm^2 glass coverslips containing a monolayer of CV-1 cells in six-welled tissue culture plates.

Coverslips were fixed on days 1, 2, 3, 4, and 5 post-inculation (PI), in 10% phosphate-buffered formalin for 30 min, placed in 100% methanol for 10 min and stained with a Giemsa-type stain. Coverslips were attached to glass microscope slides with Permount (Fisher Scientific, Fair Lawn, NJ) and examined with light microscopy.

Transmission electron microscopy. Monolayers of CV-1 cells infected with 1×10^6 merozoites from bird 100 were removed from the plastic growth surface at 8, 9, 10, or 11 d (PI) by use of a cell scraper. The cells were pelleted by centrifugation and the pellet fixed in 3% gluteraldhyde in phosphate buffer (pH 7.4) at 4 °C and processed for transmission electron microscopy. Cell pellets were then postfixed in 1% (w/v) osmium tetroxide, dehydrated in a series of ethanols, passed through two changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate and examined with a JOEL-100 CX II transmission electron microscope operating at 80 kV.

Polymerase chain reaction (PCR). Merozoites of the three isolates of *S. falcatula*-like parasites, the Cornell strain of *S. falcatula* (Lindsay et al. 1999) and SF-1A and SF2A from Argentina (Dubey et al. 2000), and SN6 isolate (Dubey et al. 1999a) of *S. neurona* were collected from infected CV-1 cells and frozen at −20 °C until used. Between 0.8×10^7 and 8.0×10^7 merozoites of each isolate were thawed and suspended in 1ml of phosphate-buffered saline (PBS). After vortex mixing, they were centrifuged in a microfuge for 1 min. The PBS was removed and the samples were resuspended in 500 μl of sterile water. A 20-μl aliquot was taken and mixed with 200 μl of InstaGene Matrix (Bio Rad, Hercules, CA). The samples were then incubated in a 56 °C water bath for 30 min. The samples were mixed and then placed in boiling water for 8 min. The samples were vortexed and centrifuged in a microfuge for 2–3 min. A 20-μl aliquot of the supernatant was used per 50-μl PCR reaction. The remaining supernatant was stored at −20 °C. Polymerase chain reaction was performed on each sample using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and a Hybaid OmniGene thermocycler. Standard PCR reaction conditions were used with the following amplification parameters: 94 °C, 5 min; 40 cycles of (94 °C, 1 min; 50 °C, 1 min 15 sec ; 72 °C, 1 min); 72 °C, 10 min. The PCR products were run on a 1% agarose gel. An 1100-bp PCR product was amplified using the specific primers JNB33 (5′-CGAACAGAGATGAGGAAAAT-3′) and JNB54 (5′-GTTGTGGTGTTGCGTGAGTC-3′) (Tanhauser et al. 1999). The PCR products, were digested separately with the restriction enzymes Hinf I and Dra I (Promega, Madison, WI). The digestion products were analyzed by electrophoresis on a 1% agarose gel with appropriate size markers. The *S. neurona* PCR product is not digested by Hinf 1 while this restriction enzyme does cut the *S. falcatula* product into 745-bp and 355-bp fragments (Tanhauser et al. 1999). The *S. neurona* product is cut into 884- and 216-bp fragments by Dra I digestion while the *S. falcatula* product is not cut by Dra I digestion (Tanhauser et al. 1999).

RESULTS AND DISCUSSION

Only a few sporocysts (total 50,000) were present in the sample obtained from the *Sarcocystis* positive opossum. Ten sporocysts measured 12 × 7 μm. Sporozoites were 6 μm long. Three of 4 budgerigars fed 5,000 sporocysts died 8, 10, and 12 DAI and one was killed 12 DAI when it was lethargic. Grossly, the lungs from all four animals were hemorrhagic or congested and *S. falcatula-like* merozoites were observed in saline smears of lungs. Schizonts were seen in sections of the lungs and these reacted with anti-*S. falcatula* rabbit serum. Sarcocysts were not seen in any of the budgerigars. Merozoites and schizonts were observed 7 DAI in a culture flask inoculated with lung tissue from bird No. 84. The two budgerigars inoculated subcutaneously with cultured-derived merozoites appeared to be lethargic on 15 DAI and *S. falcatula-like* merozoites were seen in smears and sections of lungs of birds killed 15 and 16 DAI. The two KO mice inoculated with *S. falcatula* culture-derived merozoites remained clinically normal and *Sarcocystis* infection was not found by immunohistochemical staining of their tissue when examined 71 DAI using anti-*S. neurona* and anti-*S. speeri* sera.

All six birds injected with culture-derived merozoites from bird No.100 died or were killed when clinically ill. Birds No. 150 and 152 injected with 1×10^7 merozoites died 14 DAI (Bird No.150) or were killed 22 DAI (Bird No.152) because it was ill. Birds (No.149, 151) inoculated with 1×10^5 merozoites died 19 and 20 DAI. Bird No. 154 injected with 1×10^3 merozoites died 22 DAI and the remaining bird was killed 31 DAI because it was ill. Schizonts were seen in lungs of all six birds. Sarcocysts were not seen in any of these six birds.

The two KO mice fed sporocysts remained clinically normal. No evidence of *Sarcocystis* infection was observed in their tissues.

In vitro development. Development of the isolate from bird No. 100 in cell culture is shown in Fig. 1. Merozoites were the only stages present at 1 and 2 d PI. Some merozoites contained a nucleus without a prominent nucleolus and measured 5.1 × 1.7 μm (range, 5.6–4.0 × 2.4–1.6 μm. N = 20) while others appeared to be growing in size and contained a prominent nucleolus and measured 6.9 × 2.9 μm (range, 9.6–5.6 × 5.6–1.6 μm. N = 20) at 1 d PI. Two days PI, merozoites developing into schizonts that still contained a single nucleus with a prominent nucleolus measured 9.3 × 6.0 μm (range, 12.0–8.0 × 8.0–4.0 μm) and early schizonts with a single nucleus that contained four or more prominent nucleoli were 15.5 × 10.8 μm (range, 22.4–10.4 × 16.0–8.0 μm, N = 20) (Fig. 1B). A few mature schizonts were present 3 d PI (Fig. 1A) and merozoites were often arranged peripherally around a residual body (Fig. 1H).

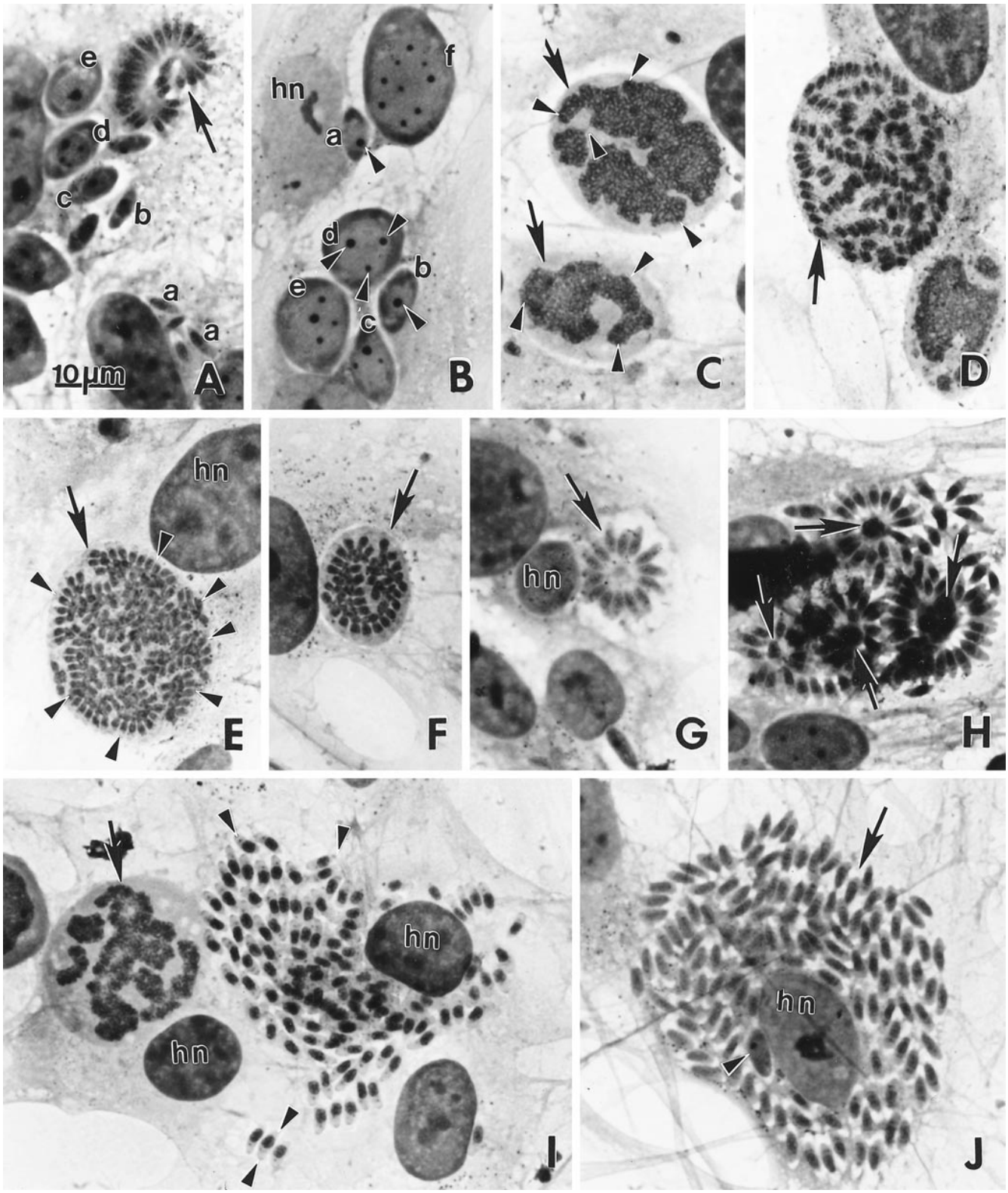

Fig. **1**. Schizogonic stages of a *Sarcocystis falcatula*-like organism in bovine turbinate cells inoculated with lung homogenate of budgerigar No. 100. Bar = 10 μm and applies to all figures. Host cell nucleus = hn. Giemsa stain. **A.** Uninucleate merozoites (**a**) and merozoites (**b–e**) differentiating into uninucleate meronts. Arrow points to a mature schizont. 3 d PI with 800,000 merozoites. **B.** Young meronts with a single nucleus (**a, b**) and with 3 (**c, d**), 4 (**e**) or 8 nucleoli (**f**). **C.** Two schizonts (arrows) with highly lobed nuclei (arrowheads). All lobes are connected. **D.** Schizont (arrow) with developing merozoites arranged in rows. **E.** Schizont (arrow) with a highly lobed nucleus giving rise to 120 or more merozoites that are arranged in at least 7 groups (arrowheads). **F.** Small schizont (arrow) with 30 developing merozoites. **G.** Small schizont (arrow) with 14 merozoites around an indistinct residual body. **H.** Five schizonts, at least four of them with distinct residual bodies (arrows). **I.**

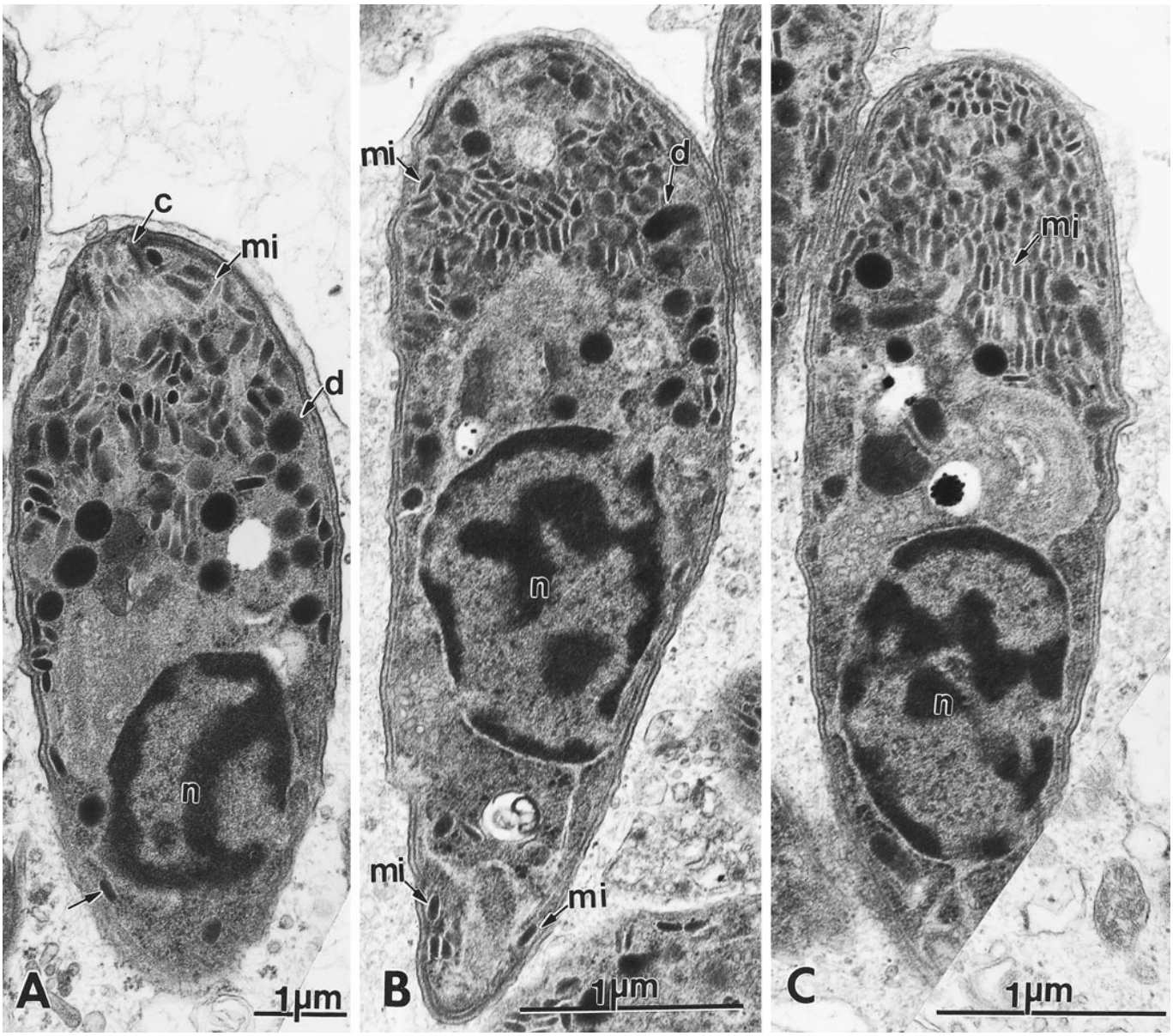

Fig. **2**. Transmission electron micrographs of merozoites of the *Sarcosystis falcatula*-like parasite in cell cultures, 11 days PI. Note conoid (c), numerous micronemes (mi), dense granules (d), and nucleus (n). Note the distribution and arrangement of micronemes. Some micronemes are present in the conoidal (in A) and the posterior end (in B). Some micronemes are arranged in rows (in B and C) and haphazardly (in A). Also note different sizes of micronemes.

Early schizonts with a single nucleus that contained four or more prominent nucleoli were 22.2 × 15.6 μm (range, 27.2–16.0 × 22.4–9.6 μm. N = 20) at 3 d PI (Fig. 1D). Schizonts with condensing nuclei were present at 3 DAI and measured 36.0 × 22.8 μm (43.2–28.8 × 25.6–20.0 μm. N = 2). Merozoites from two mature schizonts present at 3 d PI measured 5.8 × 1.6 μm (range 6.4–5.6 × 1.6 μm. N = 10). The number of merozoites in schizonts ranged from 14 to more than 100. Schizonts with condensing nuclei measured 31.0 × 22.2 μm (range, 34.4–24.0 × 26.4 × 16.6. N = 20) 4 d Pl. Merozoites present in schizonts at 4 d PI measured 5.4 × 1.6 μm (range, 6.4–4.8 × 2.4–1.6 μm. N = 20) and those present in schizonts at 5 d PI measured 5.2 × 1.9 μm (range, 5.6–4.0 × 2.4–1.6 μm. N = 20). The nuclear lobes were arranged in groups and

←

A ruptured schizont with merozoites (arrowheads) containing compact nucleus and an immature schizont (arrow) with a highly lobed nucleus. **J.** A schizont with merozoites probably developing into meronts. Note nuclei in merozoites are bigger than nuclei in merozoites in Fig. 1H and I. Also note a differentiated uninucleate meront (arrowhead).

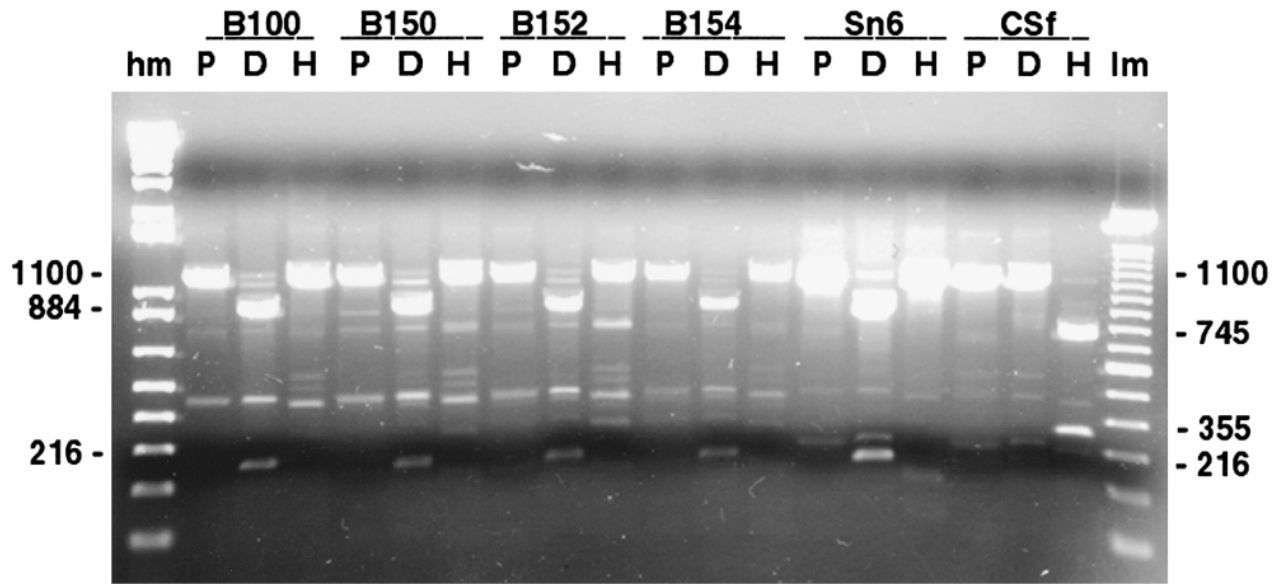

Fig. **3**. Results of PCR (P) followed by Dra I (D) and Hinf I (H) restriction enzyme digestion of merozoite DNA from *Sarcocystis falcatula*-like, S*arcocystis neurona* and *Sarcocystis falcatula* merozoites. hm = high molecular mass marker; B100 = bird 100; B150 = bird 150; B152 = bird 152; B154 = bird 154. Sn6 strain of *S. neurona*; CSf = Cornell strain of *S. falcatula*; lm = low molecular mass marker.

as many 180 nuclear lobes were identified (Fig. 1E). Merozoites formed peripherally and often were around a residual body.

The parasite from the lung of bird No. 100 divided by endopolygeny. Merozoites from cell culture contained all organelles present in coccidian parasites except rhoptries and amylopectin granules (Fig. 2). There were numerous micronemes, which varied in size and distribution. Some micronemes were arranged in rows (Fig. 2). Micronemes were present up to the posterior end but most were present at the conoidal end.

The PCR of merozoite DNA from birds No. 100, 150, 152, and 152 with primers JNB33/JNB54 each produced a 1100-bp PCR product. This product was not cut by Hinf I but was cut into 884- and 216-bp segments by Dra I (Fig. 3). An identical pattern was observed when the 1100-bp product from SN6 *S. neurona* merozoite DNA was cut with Dra I. The 1100-bp product from PCR of Cornell strain merozoites was not cut by Dra I but was cut into 745- and 355-bp products by Hinf I.

Re-examination of merozoite DNA from the SF-1A and SF-2A presumed *S. falcatula* isolates from Argentina (Dubey et al. 2000) using primers JNB33/JNB54 and both Dra I and Hinf I restriction digestion provide unexpected results (Fig. 4). Dra I digestion of the 1100-bp product produced *S. neurona*-like results giving 884- and 216-bp products. Hinf I digestion produced the expected *S. falcatula*-like digestion products of 745- and 355 bp.

Antigenically, the *S. falcatula*-like parasite isolated from *D. albiventris* is similar to *S. falcatula* isolated from *D. virginiana* because anti-*S. falcatula* serum reacted with the *S. falcatula*-like parasite in histological sections. Our limited ultrastructural studies indicate that merozoites of the *S. falcatula*-like parasite from Brazil have more micronemes than merozoites of the Cornell isolate of *S. falcatula* (Lindsay et al. 1999).

The PCR primers JNB33/JNB54 used in the present study were developed by Tanhauser et al. (1999) to distinguish sporocysts of *S. falcatula* from those of *S. neurona* in the North American opossum feces. In the present study, we used these PCR primers to investigate the identity of cell culture-derived merozoites of *S. falcatula*-like parasites from Brazil and Argentina. The *S. falcatula*-like parasites did not give the expected restriction products when the 1100-bp products were digested with Dra I and Hinf I. This indicates that these parasites are not identical to *S. falcatula* seen in North America and that the primers may not give reliable results when used to examine *Sarcocystis* parasites from South American opossums.

Tanhauser et al. (1999) characterized moleculary isolates of *Sarcocystis* sporocysts from naturally infected opossums from Florida, USA. Of the nine opossums examined, sporocysts from 5 were *S. neurona* type, 2 were *S. falcatula* type, and 2 (No. 1085, 1086) shared markers with *S. neurona* and *S. falcatula*. It remains to be determined whether our SF-1 and SF-2 isolates from *D. albiventris* from Argentina, presumed to be *S. falcatula*, are the same as isolates from opossums No. 1085 and 1086 from the North American opossum, *D. virginiana*. The parasite from bird No. 100 derived from *D. albiventris* from Brazil is a distinct species but shares characters with both *S. falcatula* and *S. neurona*. It resembles *S. falcatula* because of its infectivity to budgerigars and its reactivity with anti-*S. falcatula* serum, and to *S. neurona* in its molecular features and its development in cell culture. As said earlier, *S. falcatula* is not infective to mice and residual bodies were rarely observed in schizonts grown in vitro whereas *S. neurona* is not infectious to birds and its schizonts have a residual body. The parasite from bird No. 100 is not infective to mice and residual bodies were observed in in vitro-grown schizonts.

Marsh et al. (1999) have recently shown that the first internal transcribed spacer region (ITS-1) of the rRNA gene can be used to differentiate *S. falcatula* from *S. neurona*. They also demonstrated that there was variability in the ITS-1 of several *S. falcatula* isolates from the North American opossum and that this indicated that *S. falcatula* may be composed of a heterogeneous population. Further molecular characterizations are needed to determine the relationships between *S. falcatula* observed in the North American opossum and *S. falcatula*-like parasites isolated from South American opossums.

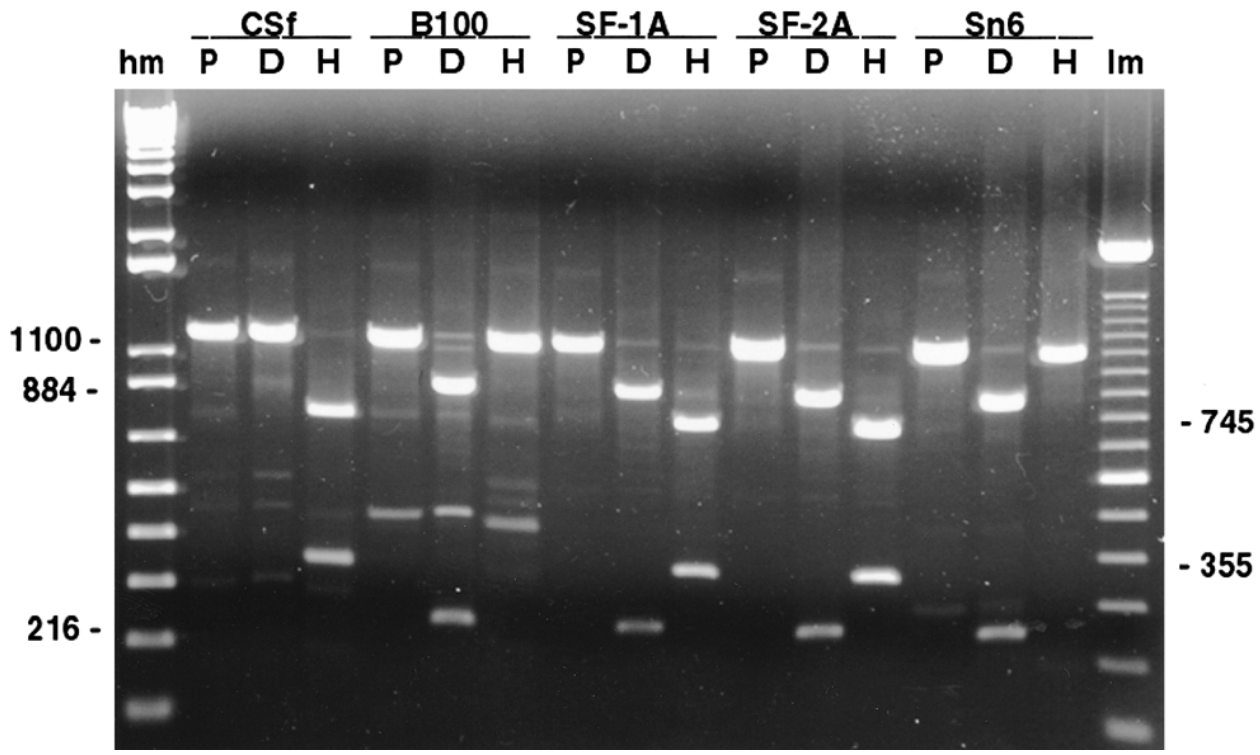

Fig. **4**. Results of PCR (P) followed by Dra I (D) and Hinf I (H) restriction enzyme digestion of merozoite DNA from *Sarcocystis falcatula*, *Sarcocystis falcatula*-like, and *Sarcocystis neurona* merozoites. hm = high molecular mass marker; Csf = Cornell strain of *S. falcatula*, B100 = bird 100; SF-1A = bird 117; SF-2A = bird 118; Sn6 = Sn6 strain of *S. neurona*; lm = low molecular mass marker.

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